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Effect of Leptin and IGFBP-3 Gene Polymorphisms on Serum IgG Level of Cattle Calves

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ABSTRACT : Leptin and IGFBP-3 are two proteins that play an important role in growth and metabolism of the animals. They are also involved in the immune function of animals and, thus, are candidate genes for the study of association with immune functions. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of these two genes was done to screen 64 crossbred (Holstein Friesian×Hariana) female calves of one year of age. From each RFLPs (fragments) three genotypes were observed. In all the RFLPs the mutant homozygotes were very less in numbers and, hence, were excluded from the least squares analysis. The serum IgG level was estimated using SRID assay. The mean level of serum IgG was 28.83±2.73 mg/ml. The effect of these identified genotypes on serum IgG level of calves at one year of age was analysed using least squares analysis. The *Hae*III RFLP-AB genotype had significantly ($p<0.05$) higher serum IgG level (31.86±3.05) than the *Hae*III RFLP-AA (25.62±2.96) genotype. There was no significant effect of leptin genotypes on the IgG level. The present results indicated a role of the IGFBP-3 gene on serum IgG level of cattle calves. (**Key Words :** Serum IgG, Leptin Gene, IGFBP-3 Gene, PCR-RFLP, Association)

INTRODUCTION

The present day scenario of animal production system suggests heavy loss in milk and beef production due to frequent occurrence of diseases to the cattle population. Animals having better performance should be selected taking care of their disease resistance capabilities also. *Bos indicus* are well known for their disease resistance capabilities (Sharma et al., 2004), while *Bos taurus* are comparatively more susceptible to diseases.

Identification of genes having important role on immune system of animals should be carried out in those populations which have larger variability in these traits. Crossbreds will have characteristics of both the species and thus the chances of variability will be higher in crossbred population.

IgG is one of the circulating antibodies that provides an important defense mechanism against diseases in healthy individuals. IgG are found in serum and in secretions from mucosal surfaces. They are produced and secreted by plasma cells that are found mainly within lymph nodes. The immune loci causally involved in susceptibility and resistance to disease are currently unknown. However, novel enabling molecular technologies promise to assist in unravelling the genetics of the host response to infectious diseases in new ways, and ultimately to improve breeding stock. Leptin and IGFBP-3 genes are the two potential candidate genes for genetic marker identification for immune traits in livestock and are located in 4th chromosome of bovines. Leptin is a hormonal protein that is secreted by adipose tissue and acts on hypothalamus to control the satiety and have role in regulation of feed intake and metabolism of the animals (Hossner, 1998; Kim and Baik, 2004). It is related to the family of class I cytokine and it can promote development and function of the immune system (Houseknecht et al., 1998). IGFBP-3 is a binding protein that binds to 90% of IGFs in the circulation to regulate later's function on growth and metabolism (Hossner et al., 1997). Besides, the IGFBP-3 has also been documented to have role in immune function of the animals (Jones et al., 1993; Oh et al., 1993; Rajah et al., 1997).

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Table 1. Primer sequences and the cyclic conditions (35 cycles) used to amplify fragment of leptin and IGFBP-3 genes

Gene/locus (Size of PCR product)	Primer sequences	Denaturation	Annealing temperature	Extension	Source
Leptin					
*Hph*I-RFLP (330 bp)	L3: 5'-GGGAAGGGCAGAAAGATAG-3' L4: 5'-AGGCAGACTGTTGAGGATC-3'	94°C; 30 s	55°C; 30 s	72°C; 45 s	Haegeman et al. (2000)
*Kpn*2I-RFLP (94 bp)	L5: 5'-ATGCGCTGTGGACCCCTGTATC-3' L6: 5'-TGGTGTCATCCTGGACCTTCC-3'	94°C; 30 s	54°C; 45 s	72°C; 30 s	Buchanan et al. (2002)
IGFBP-3					
*Hae*III-RFLP (651 bp)	P3: 5'-CCAAGCGTGAGACAGAATAC-3' P4: 5'-AGGAGGGATAGGAGCAAGTT-3'	94°C; 45 s	60°C; 45 s	72°C; 45 s	Maciulla et al. (1997)

L3, L5 and P3 are forward primers and L4, L6 and P4 are reverse primers.

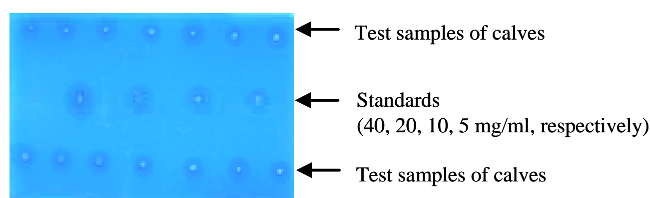

Figure 1. SRID assay showing the standards and test sample.

Polymorphic studies and nucleotide sequencing of IGFBP-3 gene has been reported in cattle (Maciulla et al., 1997; Choudhary, 2004) and in buffalo (Padma et al., 2004). The objective of the present investigation was to study the association of leptin and IGFBP-3 gene variants with the serum IgG levels in crossbred calves.

MATERIALS AND METHODS

Experimental animals

The present study was conducted on 64 crossbred cattle (½Holstein Friesian (HF)×½Hariana) maintained at Cattle and Buffalo Farm, Indian Veterinary Research Institute, Izatnagar, Bareilly, India. At one year of age, the immune system of calves is fully functional so calves of this age group were selected. Since the levels of serum IgG differ with disease conditions and with sex of the animals, the present study was carried out on healthy female calves only.

Serum and genomic DNA isolation

Blood samples were collected randomly in a 15 ml polypropylene tube containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant for genomic DNA isolation. The genomic DNA was isolated by phenol-chloroform extraction method as described by Sambrook and Russell (2001). Approximately 5 ml of blood from each animal was also collected in a separate test tube without any anticoagulant for serum isolation. The serum was extracted after clotting of blood and then stored at -70°C till further analysis.

Amplification of leptin and IGFBP-3 genes

The primers used to amplify two different regions of leptin gene and single region of IGFBP-3 gene are given in Table 1. For amplification of all the fragments PCR reactions of 25 µl were prepared separately as follows: 10 pmoles of each primer, 100 µM of each dNTPs, 1.5 mM $MgCl_2$, 2.5 µl of 10X PCR assay buffer, 80-100 ng DNA template and 1 U *Taq* DNA Polymerase. The amplification was carried out using a pre-programmed thermal cycler (PTC-200, M J Research). The cyclic conditions for amplification of both the genes are given in Table 1. The initial denaturation was done at 94°C for 5 min and final extension at 72°C for 5 min for each amplification reaction.

The 15 µl of 330 bp fragment of leptin gene was digested with 5 U of *Hph*I restriction enzyme, 15 µl of 94 bp fragment of leptin gene was digested with 5 U of *Kpn*2I restriction enzyme, while, 20 µl of 651 bp fragment of IGFBP-3 gene was digested with *Hae*III restriction enzyme, separately. The reaction was stopped by addition of loading dye in the digestion tube. The restriction fragments were observed by running the digested samples separately in 4% agarose gel.

Estimation of serum IgG concentration

Serum IgG level was assayed by single radial immunodiffusion (SRID) test reported by Mancini et al. (1965) and modified by Fahay and McKelvey (1965) and Bhat et al. (1995). The Standard curve was prepared using known concentrations of IgG. Different dilutions of IgG (40, 20, 10 and 5 mg/ml) were prepared in Tris-HCl buffer (0.1 M; pH 7.4). The 2% agar solution was prepared in Tri-HCl buffer and boiled properly. Anti bovine IgG antiserum at 1.750 ml/50 ml of gel solution was added when the gel solution cooled to 55-60°C and mixed thoroughly. The gel solution was then spreaded on defated and clean glass plate. Wells were cut in the gel after solidification and in each well 10 µl of individual concentration of IgG standards were added. The individual serum samples diluted 1:5 times were also loaded in different wells. The plate was then

Table 2. Genotype frequencies of the three RFLPs in crossbred cattle

RFLPs	Genotype	Genotype frequency
*Hph*I-RFLP	AA	0.53
(330 bp leptin gene)	AV	0.42
	VV	0.05
*Kpn*2I-RFLP	CC	0.68
(94 bp leptin gene)	CT	0.27
	TT	0.05
*Hae*III-RFLP	AA	0.65
(651 bp IGFBP-3 gene)	AB	0.32
	BB	0.03

incubated at 37°C in humidified chambers. After 17 h incubation, the antigen antibody reaction appeared in the form of ring around each well (Figure 1). The diameter (mm) of ring around each well was measured with the help of digital micrometer. The standard curve was plotted between the known concentrations of standards and respective ring diameters. The ring around each well in unknown samples were also measured and read on standard curve. The value thus obtained was the $\log_{10}$ concentration of IgG concentration. The antilog of this value was taken and multiplied with dilution factor to determine the absolute value of IgG (mg/ml) in serum.

Statistical analysis

The effect of various RFLP genotypes on serum IgG concentration was evaluated using least squares analysis technique (Harvey, 1990). Since the animals with recessive homozygotes (VV, TT and BB) were very less in number in the population, they were excluded from the analysis so as to avoid error in the results. The model used was

$$Y_{ijklm} = \mu + Sire_i + HphI\text{-}RFLP_j + Kpn2I\text{-}RFLP_k + HaeIII\text{-}RFLP_l + e_{ijklm}$$

where,

μ = overall mean of the population for the trait

$Sire_i$ = random effect of ith sire on the trait

*Hph*I-$RFLP_j$ = effect of ith *Hph*I-RFLP genotype on the trait

*Kpn*2I-$RFLP_k$ = effect of jth *Kpn*2I-RFLP genotype on the trait

*Hae*III-$RFLP_l$ = effect of kth *Hae*III-RFLP genotype on the trait

e_{ijklm} = random residual effect

RESULTS AND DISCUSSION

Digestion of the 330 bp PCR product of leptin gene with HphI restriction enzyme revealed three patterns of restriction fragments, one with 330 bp i.e. no HphI site (AA genotype); second with 330 bp, 310 bp and 20 bp (AV genotype) and third with 310 bp and 20 bp (VV genotype) in crossbred cattle. On digestion of 94 bp PCR product of leptin gene with Kpn2I restriction enzyme, three patterns of restriction fragments were obtained indicating three genotypes viz. 75 bp and 19 bp (CC genotype); 94 bp, 75 bp and 19 bp (CT genotype) and undigested 94 bp (TT genotype). The 651 bp fragment of IGFBP-3 gene also shows three types of restriction patterns, the pattern with fragments of sizes 199, 164, 154, 56, 36, 18, 16 and 8 bp was assigned as AA genotype; pattern of 215, 164, 154, 56, 36, 18 and 8 bp fragments as BB genotype and pattern of 215, 199, 164, 154, 56, 36, 18, 16 and 8 bp fragments was assigned as AB genotype. The genotype frequency of each genotype is given in Table 2. The recessive homozygotes had very low frequencies for all the three RFLPs. In our previous reports we have reported nucleotide sequencing of these two genes from HF and Hariana cattle and the restriction fragments were found to be of same length in the two sub-species of cattle (Choudhary, 2004). These sequences were submitted to the NCBI gene bank with accession numbers AY306011, AY355439, AY601888 (651 bp IGFBP-3 fragment); AY534917, AY534919, AY721089 (330 bp leptin fragment). This was the criterion to assign the fragment sizes in crossbred cattle. However the sizing for 94 bp leptin gene fragments of crossbred cattle was done on the basis of the previous reports on *Bos taurus* cattle (Acc. No. U50365).

Serum immunoglobulin G (IgG) level

The mean serum IgG level in crossbred calves of one year of age was 28.83±2.73 mg/ml as assessed by SRID test. Almost similar observations were reported by Jonic et al. (1998) in colostrums fed calves 24 h after birth. Mean IgG concentration of 12.75 mg/ml was observed in calves during first week of life (Mulvey, 1996), while, 18 mg/ml serum IgG was reported in 48 dairy calves aged up to 100 days (Norheim et al., 1985). Suri et al. (1986) reported serum IgG concentration of 25 mg/ml in healthy bulls.

Table 3. Least squares means (mg/ml) for serum IgG level in female calves of 12 months of age

Trait	Leptin gene				IGFBP-3 gene	
	*Hph*I-RFLP genotype		*Kpn*2I-RFLP genotype		*Hae*III-RFLP genotype	
	AA	AV	CC	CT	AA	AB
Serum IgG (mg/ml)	28.57±2.89 (34)	28.91±3.15 (27)	28.75±2.77 (44)	28.74±3.23 (17)	25.78±2.96 (42)	31.88±3.05* (20)

Figures in parenthesis indicate number of individuals under each genotype.

* Significant at 5% level of significance ($p<0.05$).

The serum IgG levels were analyzed for possible association with the RFLPs in leptin and IGFBP-3 genes (Table 3). The IGFBP-3 genotypes (*Hae*III-RFLP) were found to have an effect on the serum IgG concentration (p<0.05). The animals of AB genotypes (31.88±3.05 mg/ml) had higher serum IgG concentration than the animals of AA genotype (25.78±2.96 mg/ml). No such report was found in the literature scanned to compare the present findings. However, IGFBP-3 is known to have some effect on immune functions of animals. It inhibits proliferation of breast and prostate cancer cells by a cellular signaling pathway independent of IGFs (Oh et al., 1993). IGFBP-3 also induces apoptosis of p53 negative prostate cancer cell line PC3, through a novel pathway independent of either p53 or IGF-IGF receptor mediated cell survival pathway (Rajah et al., 1997). Some genes, other than the IGFBP-3 and leptin were also shown to be associated with serum IgG levels. An association of bovine neonatal Fc receptor alpha-chain gene (FCGRT) haplotypes with serum IgG concentration in newborn calves has been reported (Laegreid et al., 2002). Mallard et al. (1989) studied the influence of swine major histocompatibility genes (SLA) on variation in serum IgG concentration and found that the dd, dg and gg haplotypes had significantly higher serum IgG concentration than did the pigs of the other haplotypes. Jonic et al. (1998) suggested that the serum IgG level exceeding 30 mg/ml provided good protection to calves. The *Hae*III-RFLP locus has been shown to have significant association with the growth traits (Choudhary, 2004).

No significant association was found between the leptin genotypes (*Hph*I-RFLP and *Kpn*2I-RFLP) and serum IgG concentration (Table 3). In contrast to the present results, leptin is supposed to be a key link between the nutrient status and the immune system. There are reports that leptin stimulates cellular immunity. Exogenous leptin administration stimulates macrophage and T-cell growth and activity (Ramsay and Cranwell, 1999). From the present results it may be indicated that the role of leptin on immune system might be because of its association with some other determinant of immunity (cellular) and not with IgG. However, to rule out any association of the leptin gene with the serum IgG level, further study is needed to screen more number of RFLPs in the leptin gene and with large sample size.

In conclusion, the serum IgG level in crossbred calves of one year of age was 28.83±2.73 mg/ml. IGFBP-3 genotypes were found to have significant effect on serum IgG levels, while, leptin genotypes have not shown any significant effect on serum IgG levels.

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